II. REMARKS

Claims 17, 19-21 and 26-36 are pending in the subject application and were variously rejected. Claim 20, 26, 29, 32, 34 and 35 have been amended. Claims 19, 27, 28, 30, 31 and 36 have been canceled. New claim 37 has been added. The amendments to claims 20, 26, 29, 32, 34 and 35 and the cancellation of claims 19, 27, 28, 30, 31 and 36 have been made without prejudice or disclaimer. Applicant expressly reserves the right to file on or more continuation applications presenting the same or similar claims.

The amendments to claims 20, 26, 29, 32, 34 and 35, as well as the addition of new claim 37 are supported in the specification on page 16, lines 1 to 22; page 55, line 5 through page 57, line 17; page 63, lines 6 to 21; page 66, line 16 to page 70, line 15; Table 6 and Table 9. An issue of new matter is not raised by the requested amendments and the addition of the new claim 37. Entry of these amendment and new claim 37 is respectfully requested.

In view of the preceding amendments and the remarks which follow, Applicant respectfully requests reconsideration and withdrawal of the rejections set forth in the outstanding Office Action. Claims 17, 20, 21, 26, 29, 32 to 35, and 37 are presently under examination.

Summary of Examiner Interview

Applicants' representative thanks the Examiner for the courtesy extended during the June 12, 2007 personal interview. Applicants' representative has reviewed the Interview Summary issued by the Office. In accordance with the procedure outlined in M.P.E.P. § 713.04, the following is Applicants' summary of the interview.

1. A brief nature of any exhibits shown or any demonstration conducted: No exhibits were shown nor was any demonstration conducted.

2. An identification of the claims discussed: All pending claims were discussed.

3. An identification of the prior art discussed: Prior art was not discussed as no art, other than the assignee's copending applications that have been raised against the pending

claims.

4. A general discussion of the principle proposed amendments. Applicant's counsel discussed amending the claims to specific cancers.

5. A brief description of the general thrust of the principal arguments presented to the Examiner: During the interview, the undersigned attorney advised the Office that the grounds for rejection were improper as the evidence of record shows that the claims are commensurate with the record. Applicant's attorney discussed the reasons why the claims are enabled throughout their full scope. For example, Applicant has shown the mechanism of action of the claimed compounds, namely that they are prodrugs activated by thymidylate synthase (TS). TS is known to be overexpressed in certain cancer cells and the claims are specific to cells endogenously overexpressing TS. Applicant's counsel also discussed the evidence of record showing the claimed biological activity and the ability to reverse resistance.

6. A general indication of any other pertinent matters discussed: Applicants' attorney reviewed the subject matter of the present application in relation to the co-pending co-owned applications that have been cited by the Examiner under obviousness-type double patenting and pointed out for the Examiner's convenience the commonalities and differences among the applications.

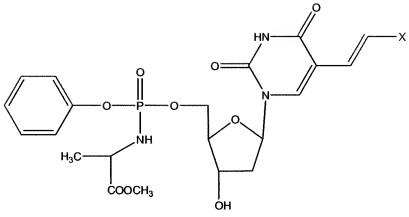
7. If appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the Examiner: Applicant's attorney offered claim amendments but the Examiner did not opine the acceptability of these offered claim amendments to remove the outstanding rejections.

Status of the Claims

After entry of the amendments, the status of the claims is as follows. Independent claim 20 is directed to a method for screening for therapeutic agents for administration in combination with (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate or (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninyl monophosphate, by contacting the candidate

therapeutic agent and (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate or (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl monophosphate with a cancer cell selected from the group consisting of a breast cancer cell, a non-small cell lung cancer cell, a rectal cancer cell, a head and neck cancer cell, a stomach cancer cell, a pancreatic cancer cell, a colon cancer cell, a liver cancer cell, a gastric cancer cell, a skin cancer cell, a bone cancer cell, a bone marrow cancer cell, a testicular cancer cell, a brain cancer cell, a lung cancer cell, a prostate cancer cell and an ovarian cancer cell, and wherein said cell endogenously overexpresses, intracellular thymidylate synthase enzyme and assaying for cell death. Claim 21 depends from claim 20 and further comprises contacting a normal cell with the candidate therapeutic agent and either (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate or (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl monophosphate. Claim 34 further limits claim 20 by requiring that the candidate therapeutic agent be contacted with the cell subsequent to contacting the cell with (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate. Claim 35 further requires that the cell be resistant to (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate. Claim 37 further limits claim 20 by requiring that the cancer cell be a colon cancer cell or a breast cancer cell.

Claim 26 is directed to a method for inhibiting the proliferation of a cancer cell selected from the group consisting of selected from the group consisting of a skin cancer cell, a bone cancer cell, a bone marrow cancer cell, a testicular cancer cell, a brain cancer cell, a lung cancer cell, a prostate cancer cell and an ovarian cancer cell, and wherein said cell and wherein said cell endogenously overexpresses thymidylate synthase by contacting the cell with an effective amount of a compound having the structure:



wherein X is Cl or I and further wherein said compound may be in any enantiomeric, diasteriomeric, or stereoisomeric form, consisting of a D-form, L-form, α-anomeric form, and β-anomeric form.

Claim 17 further limits claim 26 by requiring that the cell also be contacted with an effective amount of a compound that diminishes intracellular thymidine or purine, wherein the compound is 6-mercaptopurine, thioguanine, or 2'-deoxycoformycin. Claim 33 depends on claim 26 and requires that the contacting be conducted in vivo by administering an effective amount of the compound to a subject in need thereof.

Independent claim 29 is directed to a method for inhibiting the proliferation of a cancer cell as noted in claim 20, by contacting the cell with an effective amount of 5-Fluorouracil or Tomudex subsequent to contacting the cell with a compound, also as described in claim 20. Claim 32 depends on claim 29, and defines the compound as (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate. Claim 33 also depends on claim 29 and requires that the contacting be conducted *in vivo* by administering an effective amount of the compound to a subject in need thereof. Claim 37 further requires that in the method of claim 29, the cell be a breast cancer cell or a colon cancer cell.

35 U.S.C. § 112, First Paragraph

Claims 20, 21, 34 and 35 remain rejected under 35 U.S.C. § 112, first paragraph. The Office contends that the recitation of "assaying for cell death" is not adequately supported. The Office alleged that the disclosure only makes reference to liquid chromatography/mass spectrometry (LC/MS) in a single short paragraph bridging pages 56 and 57, but fails to provide adequate detailed guidance to disclose to one of ordinary skill how this analytical tool could be used to execute the method of claims 20, 21, 34 and 35.

Applicant respectfully traverses the rejection for the reasons which follow. Applicant directs the Office' attention to the portions of the specification which support the rejected claims and in particular, the phrase "assaying for cell death". The disclosure at page 55, beginning at line 5 and continuing through line 17 of page 57, details for assays for cell growth inhibition ("assaying for cell death"). Lines 5 through 17 of page 55 also describe cell based assays for determining the activity of compounds that can be used in combination with (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninylphosphoramidate or (E)-5-(2-bromovinyl)-2'-deoxy-5'-uridyl phenyl L-alaninyl monophosphate. Such experiments were performed as described on page 68, line 27 through page 69, line 9 and the data is presented in Tables 6 and 9. See also page 16, lines 12 to 22, for additional written description in support of the rejected claims.

Applicant further directs the Office's attention to page 64, lines 8 to 28 of the specification which describes the assay methodology referred to in the cited reference of Patterson et al. (1998) using the alamarBlue assay. A copy of Patterson et al. (1998) is enclosed for the Office's convenience.

The LC/MS method noted by the Office is described on the bridging paragraph of pages 56 and 57 also can be used to assay for cell death.

Thus, Applicant submits that the above noted description shows that Applicant had possession of the claimed invention at the time the application was filed. In view of the

preceding remarks and submission of Patterson et al. (1998), reconsideration and withdrawal of the rejection is respectfully requested.

Claims 17, 19, 26-33 and 36 also were rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter not described in the specification in a manner to reasonably convey to one of skill in the art that the inventors had possession of the claimed invention at the time the application was filed. Specifically, the Office contends that the specification does not provide enablement for treatment of the scope of neoplastic diseases encompassed by either claim 26 or 29 or of any disease condition with multiple active ingredients as specified generically in claim 29 or specifically in claim 17.

Applicant respectfully traverses noting that claims 26 and 29 require that the cancer cell whose proliferation is inhibited by the claimed method necessarily endogenously overexpresses thymidylate synthase. Conversely, cancers associated with cell proliferation which do **not** have the property of endogenously overexpressing thymidylate synthase are **not** subject to the claim. NB1011 has been disclosed to be effective for the treatment of cancer in cells based upon methods disclosed in the example on page 71 with colon cancer and in the results shown in Table 4 (page 73) for a large variety of cancer cells. Since the common property of these cell types is overexpression of thymidylate synthase, Applicant did have in his possession at the time the priority application was filed a method for treating diseases having this common characteristic. Additionally, as previously noted, the Office has not provided any objective reason why one of skill in the art would doubt efficacy of the claimed compounds against any cell which overexpresses thymidylate synthase.

The Office also cited *Ex parte Balzarini*, 21 USPQ2d 1892 (BPAI 1992)[1], as legal support for the rejection under 35 U.S.C. § 112, first paragraph. Applicant submits that the facts underlying this decision distinguish the holding and make reliance on this decision improper in support of the present rejection.

[1] See page 5 of the Office Action issued February 27, 2007.

The claims under consideration in *Balzarini supra*, were to the use of certain claimed compounds to treat retroviral infections. More particularly, the claims were directed to methods to treat or inhibit HIV infections and to treat diseased caused by HIV, most notably AIDS and AIDS-related disorders. The Office initially rejected the claims under 35 U.S.C. § 101 and § 112, first paragraph on the ground that *in vitro* evidence of record did not support the use of the compounds *in vivo*. However, unlike the facts of the present application, the Office supplied a published technical opinion supporting the contention that the *in vitro* evidence in the specification (further supported by an opinion declaration) was insufficient to establish enablement of the methods *in vivo*. In affirming the Office's rejections, the Board noted that:

> "It is apparent from this reference [Sandstrom, supplied by the Office in rebuttal of the statements made by Applicants] that in 1987 those skilled in this art did not associate successful *in vitro* treatment of HIV infected human cells with any probability of achieving success in *in vivo* treatment of this disease. While the *in vitro* testing performed on these anti-viral compounds appears to be useful as a screening tool in order to determine which of these anti-viral compounds are candidates for further testing to determine if they possess *in vivo* utility, the *in vitro* tests were not predictive of *in vivo* efficacy. As set forth on page 386 of Sandstrom in the conclusion section, the development of *in vitro* assay systems is important in this area so that "national selection of potential anti-viral compounds can be made" (emphasis added).
>
> The difficulty in concluding that a specific anti-viral compound will be useful *in vivo* in this field solely from *in vitro* testing is particularly seen from the results set forth in Sandstrom for the anti-viral compounds suramin and AZT. Suramin is a known antiviral agent which was demonstrated by Mitsuya in 1985 to protect human T-cells against infectivity and cytopathic effects of HIV *in vitro*. The authors state in the second full paragraph of this article their belief that the *in vitro* results reported in this reference provide a rationale for a "carefully-monitored experimental trial" of this anti-viral compound in patients with AIDS to determine whether suramin does inhibit HIV replication *in vivo*. Thus, even the researchers who performed the work establishing the *in vitro* efficacy of suramin against HIV infected human cells were unwilling to predict that such *in vitro* work provided a basis to conclude that this compound would have *in vivo* efficacy."
>
> *Id.* at 1895.

In contrast to the facts of *Balzarini, supra*, Applicant is neither claiming the treatment of retroviral infections and AIDS nor has the Office provided reasoned technical evidence why one of skill in the art would question the evidence of record showing how to make and use the claimed invention within the full scope of the claims.

In sum, Applicant respectfully requests reconsideration and withdrawal of the all grounds for rejection under 35 U.S.C. § 112, first paragraph.

35 U.S.C. § 112, Second Paragraph

Claims 26, 29, 32 and 36 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

The Office objected to the terms "monophosphate" and "phosphoramidate derivative of an amino acid" appearing in claim 29 on the ground the terms were insufficiently detailed to permit the ordinary practitioner to know what the particular substituents are being described. Without conceding the correctness of the Office's position and in a sincere effort to advance examination, the claims have been amended to several particular phosphoramidate compounds thereby removing these terms from the claims.

Claims 29 and 36 were objected to for failing to further define the second of the two active ingredients. Without conceding the correctness of the Office's position, claim 29 has been amended to recite 5-Flourouracil or Tomudex as the second active ingredient. In view of the amendment to claim 29, claim 36 was canceled without prejudice or disclaimer. Reconsideration and withdrawal of this ground for objection is respectfully requested.

Claims 26 and 29 were further rejected for reference to administration of a compound to a "cell" without noting that the cell was in a host in need thereof. Applicant respectfully traverses. Amended claim 26 and 29 are now directed to methods for inhibiting the proliferation of a cancer cell from the specified Markush group, by contacting the cell with an effective amount of the specified compound or compounds. As noted in the specification on page 6, lines 22 to 26, the

cells can be *in vitro* or *in vivo*, and claims 26 and 29 are intended to encompass each situation. Thus, amendment to the claims to state that the contacting is only when the cell is contained within a host or patient in need thereof would not appropriately indicate the scope of the claimed subject matter. For this reason, the rejection is improper and reconsideration and withdrawal of this ground for rejection is respectfully requested.

Claims 32 and 36 were rejected on the ground that reference was made to the term "the compound" in claim 29, but that claim 29 makes reference to a compound in two instances. In view of the amendment to claim 29 and the cancellation of claim 36, this ground for rejection has been obviated. Removal is therefore respectively requested.

In view of the preceding amendments and remarks, reconsideration and removal of all grounds for rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Obviousness-Type Double Patenting

Applicant respectfully defers responding to the grounds for this rejection until allowable subject matter has been indicated in the allegedly conflicting applications or the presently pending application.

In accordance with the duty of disclosure set forth in M.P.E.P. § 2001.06(b), Applicant notes the following co-pending applications and issued patents for the Examiner's convenience:

1. U.S. Patent No.: 6,495,553, U.S. Serial Nos.: 11/034,036; 09/789,226; and 11/627,341;

2. U.S. Patent Nos.: 6,339,151 and 6,245,750 and U.S. Serial No.: 09/782,721;

3. U.S. Patent No.: 6,683,061 and U.S. Serial No.: 10/681,418;

4. U.S. Patent No.: 7,138,388 and U.S. Serial No.: 11/516,457; and

5. U.S. Serial No.: 10/119,927.

Supplemental Information Disclosure Statement

A Supplemental Information Disclosure Statement, disclosing Patterson et al. (1998), among other references, will be filed in short order for the Office's consideration and entry into the application file.

III. CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date Aug. 27, 2007

By Antoinette F. Konski

FOLEY & LARDNER LLP
Customer Number: 38706
Telephone: (650) 251-1129
Facsimile: (650) 856-3710

Antoinette F. Konski
Attorney for Applicant
Registration No. 34,202

Attachment: Patterson et al. (1998)

Thymidine Phosphorylase Moderates Thymidine-dependent Rescue after Exposure to the Thymidylate Synthase Inhibitor ZD1694 (Tomudex) *in Vitro*

Adam V. Patterson, Denis C. Talbot, Ian J. Stratford, and Adrian L. Harris[1]

Experimental Oncology Group, Department of Pharmacy, University of Manchester, Manchester M13 9PL [A. V. P., I. J. S.], and Imperial Cancer Research Fund, Clinical Oncology Unit, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU [D. C. T., A. L. H.], United Kingdom

Abstract

The inhibition of *de novo* thymidine (dThd) synthesis by the novel folate-based thymidylate synthase (TS) inhibitor ZD1694 (Tomudex) can achieve tumor cell-specific cytotoxicity *in vivo*. However, nucleosides in the surrounding microenvironment of tumors may be used by the salvage pathway to regenerate any depleted pools, thus providing an efficient mechanism through which to circumvent the ZD1694-dependent toxicity. Anabolism of dThd to dTMP by dThd kinase (TK) is the first committed step in the dThd salvage pathway. However, dThd phosphorylase (dThdPase) can compete with TK by catalyzing the reversible phosphorolytic cleavage of dThd to thymine and deoxyribose 1-phosphate and rendering the salvaged dThd metabolically unavailable. Both TK and dThdPase are up-regulated in some tumors, and their relative importance is not fully defined. We have studied the influence of dThdPase expression on the capacity of exogenous dThd to reverse ZD1694-dependent growth inhibition and have shown that both intra- and extracellular dThdPase activity can effectively moderate dThd-rescue. This suggests that tumor levels of dThdPase may be an important factor in the outcome of ZD1694 therapy.

Introduction

ZD1694 (Tomudex) is a quinazoline antifolate TS^2 inhibitor (1). It is extensively polyglutamated by folylpolyglutamate synthase resulting in enhanced intracellular retention. Polyglutamation also serves to increase its potent inhibitory activity against TS and, thus, compromises *de novo* dThd synthesis. In the absence of salvageable extracellular dThd, a "dThd-less" state arises; the concurrent perturbations of the nucleotide triphosphate pools (2) are considered the dominant mechanism by which ZD1694 exerts its cytotoxic effects. However, preformed dThd is potentially bioavailable within a tumor mass from both the vascular supply and that released by dying cells. The high level of dThd kinase in tumor compared with normal tissues (3) implies that the salvage pathway is the preferred source of dThd for subsequent anabolism and DNA synthesis. Cellular uptake readily occurs via: (*a*) *S*-(*p*-nitrobenzyl)-6-thioinosine -sensitive and/or -insensitive cell-surface equilibrated nucleoside transporters (4); (*b*) a Na^+-associated concentrative transport process (5, 6); or (*c*) nonfacilitated diffusion. It has been demonstrated that nucleoside transport activity can be up-regulated 22- to 39-fold as a consequence of acute TS inhibition in the human bladder cancer cell line MGH-U1 (7). Such elevations in nucleoside transport function may be highly relevant when dThd availability is the potentially rate-limiting step in the salvage pathway. Thus, facilitated and nonfacilitated uptake processes could provide an effective intracellular source of dThd for the salvage pathway that could prove sufficient to circumvent the cytotoxic impact of ZD1694-mediated TS inhibition.

dThdPase (EC 2.4.2.4) is the first catabolic enzyme in the dThd salvage pathway, and, thus, represents a potential mechanism by which dThd can be rendered unavailable for subsequent phosphorylation by dThd kinase. dThdPase catalyzes the reversible phosphorolytic cleavage of dThd and deoxyuridine to their respective bases and deoxyribose 1-phosphate (8, 9). Although both reactions are reversible, with an equilibrium constant close to one, the limited availability of deoxyribose donors favors the phosphorolytic over the transferase reaction. The utilization of thymine in cells is generally less than 1% of dThd incorporation, which supports the evidence that dThd catabolism dominates thymine anabolism (8, 10, 11).

Expression of dThdPase is elevated in many malignant tumors, but a wide range of activities have been reported (review, Ref. 12). We have previously confirmed this heterogeneity of dThdPase activity in a sample group of breast tumor cytosols prepared from primary excision biopsies (13, 14). It is conceivable that the observed elevations in dThdPase activity could contribute to the efficacy of ZD1694 *in vivo*.

To evaluate the potential role of elevated dThdPase activity *in vitro*, the human breast carcinoma cell line MCF-7 was transfected with human dThdPase cDNA, and a stable clone TP-4 was generated. The wild type and clonal line were tested for their response to TS inhibition with various "pulses" of dThd rescue during and/or after ZD1694-exposure. The addition of exogenous *Escherichia coli* dThdPase was also examined to establish the relative contributions of intra- *versus* extracellular dThdPase activity. Our data demonstrate that a complete rescue of the parental line occurs if exogenous dThd (0.3–3 μM) is present during the 48-h ZD1694 exposure and for at least 48 h afterwards. In contrast, the same 96-h rescue was almost totally ineffective in the transfected cell line TP-4, which overexpressed intracellular dThdPase. Furthermore, when comparative activities of exogenous *E. coli* dThdPase (initial seeding density, 10^4 TP-4 cells) were included during the "window" of dThd rescue, this phenomenon could be reproduced almost as effectively in the parental line. These data suggest that intra- or extracellular dThdPase activity, when present during initial ZD1694 exposure, can significantly reduce the bioavailability of dThd and, therefore, contribute to the cytotoxic impact of TS inhibition.

Materials and Methods

Chemicals. ZD1694, a gift from Zeneca (Alderley Edge, United Kingdom) was dissolved in 0.4 M sodium bicarbonate and stored in aliquots at 4 C, protected from light. Cell culture-grade dThd, *E. coli* dThdPase, and all of the other chemicals were purchased from Sigma (Poole, United Kingdom).

Cell Lines. Human MCF-7 breast cell lines (passage 52–75) and the clone TP-4 were grown in DMEM. (All of the media were prepared at Imperial Cancer Research Fund Laboratories, Clare Hall, United Kingdom.) The medium was supplemented with 10% (v/v) FCS and 2 mM glutamine. Cells were grown at 37°C in a 100%-humidified incubator with a gas phase of 5% CO_2. Cultures were routinely screened as negative for *Mycoplasma*.

Transfection of dThdPase cDNA into MCF-7 Cells. Transfection and isolation of dThdPase clones have been described elsewhere (14). Briefly, pSV^{neo} plasmid vector containing full-length dThdPase DNA was introduced into MCF-7 cells by electroporation. Stable transfectants were selected by long-term incubation in Geneticin.

Quantification of Drug Sensitivity. The MTT-proliferation assay was used to determine the dose-response curves of the parental and clonal cell lines as described previously (15). The cells were seeded at an initial density of 10^4

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[1] To whom requests for reprints should be addressed, at ICRF Clinical Oncology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom.

[2] The abbreviations used are: TS, thymidylate synthase; dThd, thymidine; dThdPase, dThd phosphorylase; 5-FU, 5-fluorouracil.

Table 1 *Parental MCF-7 cell line*

Day 1[a]	Day 2[a]	Day 3	Day 4	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	IC50 (nM)[b]
		+[c]	+	+	+	+	+	+	+	+	6.0
		+	+	+	+	+	+				9.6
		+	+	+	+	+					8.8
		+	+	+	+						7.4
		+	+	+							6.7
		+	+								5.9
		+									6.4
		+	+	+	+	+	+	+	+	+	6.0
			+	+	+	+	+	+	+	+	5.4
+	+					+	+	+	+	+	5.8
+	+	+									58.0
+	+	+	+								103
+	+	+	+	+							>10,000
+	+	+	+	+	+						>10,000
+	+	+	+	+	+	+	+				>10,000
						+	+	+	+	+	>10,000

[a] All of the plates were treated with 1–10,000 nM ZD1694 for an initial 48-h period.
[b] IC50 (where determinable) is the mean of three independent experiments.
[c] +, 24-h treatment with 1.0 μM dThd.

cells/well. IC50 values were determined relative to the control wells, which contained no drug. After initial evaluation, all of the ZD1694 exposures were for 48 h. Exogenous dThd or dThdPase additions were varied as described in the "Results" section. Cells were allowed to grow for a total of 12 days.

Preparation of Cell Lysates. Cell lines were harvested, washed, and sonicated using a MSE Soniprep 150 three times for 5 s (nominal frequency of 23 KHz and an oscillation amplitude of 5–10 μm). Sonication was performed in 50 mM Tris-HCl and 0.15 M NaCl buffer (pH 7.4) at 4°C. Suspension was centrifuged at 10,000 × *g* for 15 min (4°C). Supernatants were stored in liquid N_2 until required.

dThdPase Activity Determinations. Enzyme activity determinations were conducted for the parental MCF-7 and cloned TP-4 line *in vitro*. The assay conditions have been described previously (16). Briefly, lysates were incubated for 16 h at 37°C in 10 mM dThd and 50 mM K_2PO_4 (pH 7.4). The reaction was terminated by the addition of 0.7 ml of ice-cold 0.5 M NaOH to 0.3 ml of reaction mixture to produce a final solution pH of 13.3, and the conversion of dThd to thymine was monitored spectrophotomerically at 300 nm. Optical densities were related to the standard plots for known thymine concentrations. The protein content of the cell lysates were determined using the Bio-Rad protein dye-binding assay and quantified against a high-grade BSA protein standard. dThdPase activity is expressed as nmol substrate converted/mg total cytosolic protein/h.

Influence of ZD1694 Exposure on [^{3}H]dThd Uptake. Subconfluent monolayers of MCF-7 parental and TP-4 cells were incubated for 4 h with or without ZD1694 (10–100 nM) in serum-free medium; 0.3 μM [^{3}H]dThd (20 μCi/mmol) was added for 30 min, after which the medium was rapidly aspirated. Cells were washed three times in ice-cold calcium- and magnesium-free phosphate buffered saline-A, harvested by trypsinization, and pelleted (4°C). The supernatant was discarded and 0.5 ml of ice-cold 0.4 M perchloric acid was added to the pellet and mixed thoroughly. After standing on ice for 30 min, the acid-insoluble material was pelleted at 1600 × *g* for 20 min (4°C). Supernatant, containing the acid-soluble nucleotide pool, was spotted on silica gel 60 TL plates, and dThd and thymine were separated in chloroform:acetone:methanol (17:3:1). The solvent front was run to the top of the TLC sheet, and spots corresponding to dThd and thymine were visualized under UV illumination (254 nm), recovered, and dispensed into scintillation vials for counting. The acid-insoluble pellet was washed twice with 0.2 M perchloric acid and solubilized in 1 ml of 0.3 M KOH before transfer to scintillation vials.

Results

dThdPase Activity Determinations. The dThdPase activities of the parental and TP-4 MCF-7 cell lines and their relationship to activities found in primary breast biopsies have been reported previously (13, 14). Basal activity of the parental MCF-7 cell line was 38.2 ± 5.9 as compared with 3383 ± 133 nmol thymine released/h/mg lysate protein (37°C) for the TP-4 cloned line. This represents a 88-fold elevation in dThdPase activity. Doubling times for the two cell lines were not significantly different *in vitro*.

Optimization of dThd Rescue in the MCF-7 Wild-Type Cell Line. Initial experiments were conducted on the parental line to determine the most effective schedule of dThd (1.0 μM) rescue during and/or after the 48-h exposure to ZD1694. These data are summarized in Table 1. The addition of dThd during the 48-h ZD1694 exposure was essential for effective rescue. If the rescue was initiated only on removal of the drug and continued for the remaining 10 days of growth, a modest shift in IC50 from 6.0 to 9.6 nM could be achieved. However, if a delay of 24 h was introduced before the dThd addition, rescue was totally ineffective. Thus, the ability of exogenous dThd to rescue

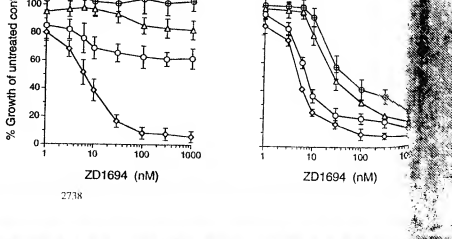

Fig. 1. Representative ZD1694 dose-response curves for the MCF-7 parental cell line (*A*) and the TP-4 clonal line (*B*), which expresses an 88-fold elevation of dThdPase activity relative to the parental line. All of the ZD1694 treatments were for 48 h in the presence of dThd, and dThd was present for an additional 48 h after the removal of the drug. Concentrations of exogenously added dThd are (◇) 0 μM, (○) 0.3 μM, (△) 1.0 μM, and (▽) 3.0 μM. Cells were allowed to grow for an additional 8 days after the removal of dThd (total growth time, 12 days).

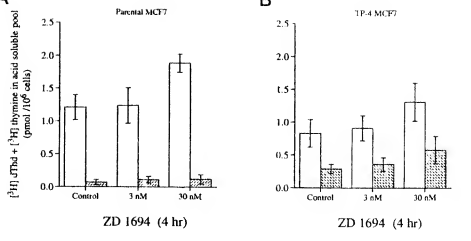

Fig. 2. The influence of ZD1694 exposure on [^{3}H]dThd uptake in MCF-7 parental cells and TP-4 cells. Cultures were incubated for 4 h with or without ZD1694 (3 and 30 nM, respectively) in serum-free medium. Then 0.3 μM [^{3}H]dThd (20 μCi/mmol) was added for 30 min, after which the medium was rapidly aspirated. Cells were harvested (4°C) and [^{3}H]dThd (*clear bars*) and [^{3}H] thymine (*hatched bars*) were separated at 4°C and counted

parental MCF-7 cell line from ZD1694 toxicity was found to be highly schedule-dependent, with the initial 72–96 h "window" being critical.

If the dThd rescue was begun at the time of ZD1694 addition and various time points, almost complete reversal of growth delay was achieved within 96 h. More extended periods of rescue did not improve the rescue phenomenon any further under these drug-exposure conditions. Therefore, the addition of exogenous dThd for 96 h, during and after the 48-h ZD1694 exposure was considered optimal, and this schedule was adopted for subsequent experimentation. In parallel control experiments, the biologically inactive isomer of dThd, α-dThd, was unable to rescue cells from ZD1694 exposure under any conditions (data not shown).

Influence of Elevated Intracellular dThdPase Activity on dThd Rescue. In contrast, experiments conducted using the clonal line TP-4 demonstrated that the presence of elevated dThdPase activity could very significantly modulate this "dThd rescue" phenomenon. By using the previously optimized schedule of drug exposure (48 h) and dThd rescue (96 h), MCF-7 wild-type and TP-4 cell lines were rescued with various concentrations of exogenous dThd (0.3–3.0 μM). In the absence of dThd, dose-response curves were similar for the two cell lines, with IC50 values in the low nM range (Fig. 1, *A* and *B*). The TP-4 line was modestly but consistently ($P = 0.027$) more sensitive than the parental line (3.5 and 6.0 nM, respectively) under these conditions, perhaps indicating the presence of low levels of dThd in the FCS medium supplement.

The addition of 0.3 μM dThd (96 h) resulted in a significant modulation of the dose-response curve for the parental line, such that an IC50 value was no longer determinable (Fig. 1*A*). In contrast, 0.3 μM dThd rescue in the TP-4 cell line only shifted the IC50 value from 3.5 to 8.0 nM (Fig. 1*B*). Increasing concentrations of exogenous dThd (1.0 and 3.0 μM) were more effective at reversing the ZD1694-dependent toxicity in both cell lines. However, the rescue was far superior in the parental line. Indeed, no significant inhibition of growth could be achieved in the parental line at either 1.0 or 3.0 μM dThd, whereas IC50 values for the TP-4 line were 26 and 88 nM, respectively. This represents a very significant modulation of rescue at physiologically relevant dThd concentrations and suggests that, within this *in vitro* model, elevated dThdPase activity need only be present during the initial period of TS inhibition to compromise any dThd-dependent rescue phenomena.

Uptake and Metabolism of [^{3}H]dThd in ZD1964-Treated Cells. After a 4-h treatment at 3 or 30 nM ZD1694, parental and TP-4 cells were pulsed with 0.3 μM [^{3}H]dThd for 30 min before harvesting and acid extraction. Total uptake of [^{3}H]-label was similar in both cell lines under comparable conditions. Consistent with the impact of exogenous dThd on the modulation of the dose-response curves of the parental (but not the TP-4 cell line), a greater proportion of the initial dThd uptake was metabolized to thymine. At 30 nM ZD1964 pretreatment, 29% (0.57 pmol/10^6 cells) of the total salvaged dThd was rendered metabolically unavailable to relieve the acute TS inhibition in the TP-4 cells, compared with just 3.6% (0.11 pmol/10^6 cells) in the parental cell line (Fig. 2). Therefore, the presence of intracellular dThdPase activity could modify the extent to which dThd was available via the nucleotide salvage pathway.

Comparative Roles of Intra- and Extracellular dThdPase Activity. To examine whether the presence of extracellular dThdPase activity would be sufficient to mimic this effect seen for intracellular expression, increasing quantities of *E. coli* dThdPase were added exog-

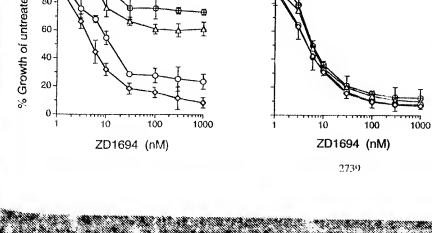

Fig. 3. Representative ZD1694 dose-response curves for the MCF-7 parental cell line. All of the ZD1694 treatments were for 48 h in the presence of dThd and for 48 h after the removal of the drug. Concentrations of exogenously added dThd are (◇) 0 μM; (○) 0.3 μM; (△) 1.0 μM; and (□) 3.0 μM. *E. coli* dThdPase was also present during the period of dThd-rescue at specific activities equal to 10^4 TP-4 cells (total activity of 20.6 nmol thymine released/h) (*A*) and 10^5 TP-4 cells (*B*). Cells were allowed to grow for an additional 8 days after the removal of dThd ± *E. coli* dThdPase (total growth time, 12 days).

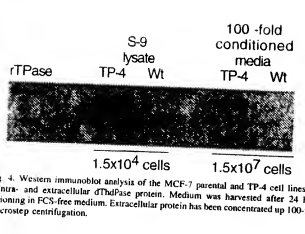

Fig. 4. Western immunoblot analysis of the MCF-7 parental and TP-4 cell lines for both intra- and extracellular dThdPase protein. Medium was harvested after 24 h of conditioning in FCS-free medium. Extracellular protein has been concentrated up 100-fold by macrostep centrifugation.

enously. Extracellular addition of dThdPase at a total activity like that found in 10^4 TP-4 cells (20.6 nmol thymine released/h at 37°C) was sufficient to modulate the salvage capacity of 0.3 μM exogenous dThd in the parental cell line, whereas dThdPase activity equivalent to 10^5 cells (206 nmol/h) ablated rescue by 3 μM dThd *in vitro*. The addition of *E. coli* dThdPase in the absence of exogenous dThd had only a modest influence on ZD1694 toxicity, consistent with the presence of some dThd in the undialyzed FCS supplement. Representative dose-response curves are shown in Fig. 3, *A* and *B* (compare with Fig. 1*A*).

These data suggest that the delivery of catalytically active dThdPase to the cell surface may enhance the efficacy of ZD1694 *in vivo* and may, therefore, be an appropriate enzyme to consider for antibody-directed therapy in this context.

Western Blotting Analysis of Intra- ***versus*** **Extracellular dThdPase Protein in Conditioned Medium.** Analysis of the relative proportions of dThdPase protein in the intra- and extracellular fractions after 24-h medium conditioning was conducted by Western blotting (Fig. 4). Densitometric analysis of total dThdPase protein in cell extracts *versus* conditioned medium that was concentrated 100-fold by Macrostep centrifugal concentration (10 kDa cutoff) confirmed the presence of at least a 10,000-fold excess of intracellular dThdPase protein in the TP-4 cell line. This suggests that the background levels of extracellular dThdPase are not contributing significantly to the metabolism of exogenous dThd, and, therefore, in the absence of exogenously added *E.Coli* dThdPase, intracellular metabolism dominates.

Discussion

The observation that ZD1694 toxicity is totally ablated by the presence of salvageable dThd is consistent with the primary mode of action being TS-inhibition. Thus, despite a complete block in *de novo* dThd synthesis, dThd in the surrounding environment can be used by the salvage pathways to regenerate the depleted nucleotide pools, ensuring continued DNA synthesis and repair. Critically, nucleosides are present in human plasma at concentrations sufficient to fulfill a salvage function and may also be available locally from the nucleic acids of dying cells within a tumor mass (17). Considering this together with evidence that the contribution of salvage flux is greater than that of *de novo* synthesis in neoplastic tissues and that the inhibition of *de novo* synthesis leads to even higher salvage activity (7), one might speculate that any approach that modulates the availability of exogenous pyrimidine nucleotides should significantly enhance ZD1694 toxicity. Consistent with this proposal, we have demonstrated that the presence of elevated dThdPase activity, the first enzyme in the dThd salvage pathway, can markedly compromise the intracellular bioavailability of salvaged dThd.

Significant evidence has accumulated that dThdPase is markedly elevated in many solid tumors including breast, esophageal, pancreatic, lung, bladder, ovarian, gastric, and colorectal cancers (12). However, the analysis of dThdPase activities in primary biopsy samples and/or the immunostaining of tumor sections have demonstrated a significant intertumoral variability. Our *in vitro* data suggest that this heterogeneity may be a factor in the efficacy of ZD1694 treatment and therefore, imply that approaches aimed at rational patient selection based on "enzyme-profiling" of tumor tissues could target those individuals most likely to benefit from treatment with ZD1694. Several studies have indicated that high dThdPase expression is associated with poor prognosis (12, 16, and references therein). We have reported recently that patients treated with adjuvant cyclophosphamide, methotrexate, and 5-FU for node-positive breast cancer had a better prognosis if dThdPase was elevated in their tumors (18). Methotrexate cytotoxicity, and perhaps 5-FU cytotoxicity, can be potentiated by preventing dThd-rescue (19), which suggests that the influence of dThdPase on therapy with cyclophosphamide, methotrexate, and 5-FU reflects a potential for manipulation of the salvage pathway *in vitro*. Perhaps therapies that are aimed at exploiting this prognostically unfavorable tumor characteristic may prove to be of value. Alternatively, approaches that are aimed at inducing tumor dThdPase activity, such as IFN-α and IFN-γ treatment (11), may enhance the response to ZD1694 treatment.

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